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USE OF VIGNA RADIATA LECTIN GENE IN DEVELOPMENT OF TRANSGENIC *BRASSICA JUNCEA* RESISTANT TO APHIDS

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ABSTRACT

Mustard (Brassica juncea L. Czern) is a major oilseed crop with great economic value being cultivated on more than six million hectares in the Indian subcontinent. Due to increase in demand of edible oil the cultivation of this crop has gained elevated importance. Lipaphiserysimi (Kalt.) is the serious insect pest leading to yield losses as high as 97.6 %. Plant lectins especially from legumes have been implicated in insecticidal action against hemipteran insects. Vigna radiata lectin gene was directionally cloned under phloem specific rolC promoter in a binary vector and introduced to cotyledonary petioles of Brassica juncea cv. Varuna through Agrobacterium-mediated transformation method. Molecular analysis confirmed the integration of transgene into Brassica juncea cv.Varuna. The presence of Vigna radiata lectin gene and nptIIconfirmed the integration of transgene. The developed transgenic plants of Brassica juncea will have more resistance to Aphids, reducing losses and increasing the production.

KEYWORDS: Lipaphiserysimi, Plant lectins, Brassica juncea cv.Varuna

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Original Article

INTRODUCTION

Agricultural plants continuously face an array of pathogens and adverse environmental factors which are major causes of limiting crop production. Mustard (*Brassica juncea*) belongs to the *Cruciferae* family and being an important oil seed crop grown in many countries across the world. In India, mustard produces 28.6% of total oilseed production and has been projected to provide for 41% (14 million tons) of the country's demand by the year 2020 (Shekhawat*et al*., 2012). Unfortunately, the productivity of this crop has been hampered by several biotic and abiotic factors (Grover *et al*., 2003). Among various pest the mustard aphid, *Lipaphiserysimi* (Kaltenbach) is a serious pest of mustard in India and other tropical regions in the world and inflecting as high as 97.6% yield loss in different varieties (Patel *et.al* , 2004). Since, the insecticides and fungicides for the control of this pest adversely affect the seed quality, pollute the environment and also result in the evolution of resistant biotypes of insects. Thus, an efficient crop protective strategy can stop such a destructive cycle. Non availability within primary gene pool of *Brassica* has limited breeding progress for disease and insect resistance. Therefore, developing aphid resistance in mustard through genetic engineering technique assumes high significance.

Transgenic attempt to address this issue has been encouraging recently.Plant lectins were used to confer resistance against *hemipteran* insect pests (Schuler *et al*. 1998). They are highly antinutritional and toxic to various phloem feeding insect pests which were documented earlier by artificial diet based insect bioassay

(Powell*et al.*, 2001). Several carbohydrate-binding plant lectins, i.e. snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) (Gatehouse *et al* ., 1996; Shi *et al*., 1994; Hilder*et al*., 1995; Powell *et al*.,1995a,b; Sauvion*et al*., 1996; Rao*et al*., 1998; Stoger*et al* ., 1999; Foissac*et al*., 2000; Nagadhara*et al* ., 2004), wheat germ lectin (wheat germ agglutinin, WGA) (Kanrar*et al* ., 2002) and concanavalin A (Con A) (Gatehouse *et al* ., 1999), have been reported to have detrimental effects on homopteran pests.

The insecticidal activity of carbohydrate-binding plant lectins against different insects belonging to the orders *Coleoptera*, *Diptera*, *Lepidoptera* and *Homoptera* have been well studied (Gatehouse *et al.*, 1995; Schuler *et al*., 1998; Carlini and Grossi-de-Sa., 2002). Recently, another mannose-binding 25-kDa homodimeric lectin, *Allium sativum* leaf lectin (ASAL), isolated from leaves of *A. Sativum* and having a high degree of sequence similarity to GNA, has been shown to affect the survival of some sucking pests, namely red cotton bug, mustard aphid and green leaf hopper of rice (Bandyopadhyay*et al.*, 2001; Roy *et al.*, 2002; Majumder*et al.*, 2004). The plant lectin genes can be genetically manipulated to produce transgenic mustard crops expressing resistance to aphids and in turn benefit the environment by being an alternate to the use of chemical pesticides/insecticides. Therefore isolated legume lectin gene which is free from IPR issue can be used for development of transgenic and can be commercialized (Koundal *et al*., 2003).

The present study describes the development of transgenic *Brassica juncea* cv. Varuna through agrobacterium mediated transformation using cotyledonary petiole as explants with insecticidal *Vigna radiata* lectin gene under the control of phloem specific (rolC) promoter against aphid's resistant.

MATERIAL METHOD

Vector Construction

Vigna radiate lectin gene (GenBank Accession no. HM348715) cloned in pGEMT-Easy vector was PCR amplified using gene specific primers designed with *Eco*RI and *Kpn*I restriction sites in the forward and reverse primers. The amplified product was purified using PCR amplification kit (QIAquick Gel Extraction Kit). The binary vector pOREO4 with rolC promoter and purified amplicon were restricted with *Eco R*I and *Kpn* I restriction enzymes. 100ng of the linearized binary vector was ligated with 60 ng of the eluted gene fragment using T4 DNA ligase at 4°C for overnight. The competent cells of *E.coli* strain DH5α was transformed with ligated product and plated on LA medium plates supplemented with kanamycin (50µl/ml) and kept ovemight at 37°C±1°. The transformed colonies were confirmed for the presence of the desired gene by colony PCR. The plasmid DNA of the confirmed clone was mobilized into *Agrobacterium* strain GV3101 by freeze thaw method.

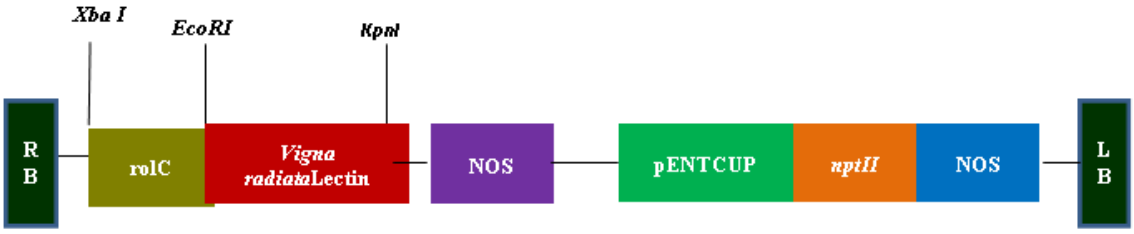

Figure 1: Diagrammatic representation of *Vigna radiata* Gene in Binary Vector pOREO4

MATERIAL METHOD

Transformation of *Brassica juncea* cv. Varuna using *Vigna Radiata* Lectin Gene

Plant Material

The seeds of *Brassica juncea* cv. Varuna were washed with 1% teepol, surface sterilized with 0.1% mercuric chloride and five times washed with sterile water. The seeds were sown on the ½ MS medium (Murashige and Skoog, 1962) in tubes and kept for germination in dark for 48 h followed by incubation for 72 h under 16 h light and 8 h dark period. All seeds were grown at 25 ±1°C under continuous light (25 µmol/m2/s).

Co-Cultivation

The cotyledonary petioles were excised from five day old seedlings and preculturedon MS medium augmented with BAP (2µg/L) and 2, 4-D (0.2µg/L). A single colony of transformed *A. tumefacien*s strain GV3101 containing *Vigna radiata* lectin gene cloned under the control of phloem specific (rolC) promoter was grown in10 ml of YEM medium supplemented with 25 µg/L of rifampicin and 50 µg/L kanamycin at 28 ± 1ºC for overnight at 200 rpm. Next Day, the culture was centrifuged at 3,000 rpm for 2 min and gently suspended in 50 ml MS medium supplemented with phytohormones. The preculturedcotyledonary petioles were incubated with 50 ml *Agrobacterium* suspension for 30 min with occasional gentle shaking under dark conditions. The infected explants were blotted dry on sterile tissue paper and plated on the MS medium supplemented with BAP (2µg/L) and 2, 4-D (0.2µg/L) and kept in dark for 2 days at 25±1˚C.

Selection and Regeneration

After 2 day of co-cultivation the cotyledonary petioles were washed in washing medium (sterilized distilled water with 250 µg/L cefotaxime) three times for 5 minutes to remove the bacteria attached to the explants and then blotted dry on sterile tissue paper. The explants were placed in *Agrobacterium*-elimination medium (MS media supplemented with BAP (2µg/L), 2,4-D (0.2µg/L) and 250 µg/L cefotaxime).After three days, the explants were transferred to a selection medium (MS media with BAP (2µg/L), 2,4-D (0.2µg/L), 250 µgL cefotaxime and kanamycin 30 µg/L)at 25 ± 1˚C for 10 days intervals with 16 h light: 8 hphotoperiod till the shoots emerged. Regenerated shoots were further subcultured once or twice on the same medium. After six weeks multiple shoots possessing leaves and apical buds were excised then transferred to root-induction medium containing ½ MS+ 2,4D (0.2µg/L) + kanamycin (30µg/L).The transfomation efficiency was calculated as the percentage of total number of kanamycin-selected shoots out of total number of *Agrobacterium*-infected explants. The putative transformants with well-formed root systems were gently removed from the culture tubes and the medium was washed away from the roots using lukewarm water. The well-developed plantlet were acclimatized, hardened and shifted to pots with thoroughly wetted autoclaved soil and agropeat in the ratio of 2:1 covered by perforated plastic bags to maintain humidity for 10 days. This process was repeated for 1 week for a gradual acclimation. The well grown putative transformants were then shifted to glasshouse of National Phytotron Facility, IARI, New Delhi for further growth. The untransformed wild type plants were also regenerated under the same conditions without antibiotics. The putative transformants were regularly monitored and T1 seeds were collectedfor further analysis.

Molecular Analysis of Transgenic *Brassica juncea*cv. Varuna

PCR

Genomic DNA was isolated from leaf tissue of transformed and non transformed *Brassica juncea* cv. varuna by using cTAB method (Murray and Thomsom, 1980). The primer pair for amplification of *Vigna radiata* lectin gene was

MblF 5'gaattcatggcttctcttcaaaccca 3' and MblR 5' ggtaccctatgcatctgcagcttgct 3'. For amplification of selection marker gene, the forward primer was nptIIF 5' caagccaagcacagaattga 3' and reverse primer was nptII R 5' gcatctccatgtgtcaccac 3'. The amplified PCR products wereseparatedon 1% agarose gel electrophoresed at 40 volt for 3 h and visualized using a gel documentation system.

Semi-Quantitative RT-PCR Analysis

To perform Reverse Transcription PCR, RNA was isolated using TRIzol® reagent(Life Technologies™, Invitrogen, USA). 2µg of total RNA was used for reverse transcription PCR primed with gene specific primers in amplification of *Vigna radiata* lectin geneand *nptII* marker gene in both orientations using one step reverse transcriptase PCR kit, (QIAGEN one step RT-PCR Kit). As a reference gene β Actin gene of *B. juncea* was used. The primersequences used for β Actin forward amplification was ActinF 5'ctcacgctatcctccgtctc 3' and for reverse wasActinR 5' ttctccaccgaagaactgct 3'.

Segregation Analysis

PCR-positive plants was segregated and molecularly analysed for T1 progeny. T1 generation seeds were surfaced sterilized with 0.1% w/v $Hgcl_2$ and allowed to germinate on ½ strength MS medium containing 50 µg/L kanamycin in plant growth chamber to determine the pattern of inheritance of *Vigna radiata* lectin gene. The surviving green plantlets were transferred to soil and grown under greenhouse condition for further development.

Southern Blot Analysis

Southern blot analysis was carried out to confirm the transgene integration according to the protocol of (Kochert*et al*., 1989) with slight modifications. Genomic DNA was isolated and purified from young green leaves of 1-month-old putative transformants and non transformants plants according to cTAB method. Approx.10 µg of genomic DNA from putative transformants and non Transformants were digested with *EcoR*I, separated in 1 % (w/v) agarose gel and blotted on to positively charged nylon membrane (Hybond-N+). The PCR purified fragment DNA of *Vigna radiata* lectin gene was used for [α-^{32} P] dCTP radiolabel probe prepration using hexalable DNA labeling kit (MBI, Fermantas). The U.V cross-linked blot was pre-hybridized for 15 minutes at 65ºC using Amersham Rapid-hyb buffer. The radiolabelled probe was then added to the Rapid-hyb buffer for 2 hrs at 65°C with shaking. The membranes were then washed thoroughly using 2XSSC, 0.1% SDS at room temperature for 45 min and at 65 ºC for another 45 min using 0.1% SSC, 0.1% SDS and then exposed to Kodak X-ray film and stored at -80 ºC for 7 days and subsequently developed.

Aphid Bioassay

Aphid bioassay was performed to assess the affectivity of T1 putative plants against aphids. *Lipaphiserysimi were* collected from mustard plants growing in the field at IARI Campus, New Delhi. Leaves were collected from transgenic as well as non transformant plants with their cut ends sealed with wet cotton and placed on petriplates.25 aphids were placed over each leaf (transgenic as well as control plants). For each transgenic line as well as control, three replicates were maintained. Experiment was carried out under controlled conditions (in the germinator) with 75% relative humidity,at 16h: 8h light: dark photoperiodand 23±1 ºC temperature. The aphid's survival was monitored at an interval of 24 h for 7 days. The mortality rate of aphids was assessed by the total number of insects survived on individual T1 putative plant leaf and non transformant plant leafat the end of the experiment.

The mean data per plant was expressed as the percentage of total surviving aphids. Statistical analysis of the aphid data was performedto test for significance of any differencesbetween treatments, with a rejection limit of$P \leq 0.05$.

RESULTS AND DISCUSSIONS

Transformation of *Brassica juncea* cv. Varuna using *Vigna radiata* Lectin Gene

Transgenic *Brassica juncea* were produced by infecting cotyledonary petioles with engineered *Agrobacterium tumefaciens*harbouring phloem specific promoter (rolC) for expression of *Vigna radiata*lectin (Figure 2A). Green calli were induced from the co-cultivated explants which differentiated and turned green-compact within 15 days on selection medium (Figure 2B). An average of 2-6 green shoots with 2-3cm in length were produced from each explants, which elongated in shoot elongation media (Figure 2C). The strong branched shoots emerged out within 12-15 days.The multiplied shoots were excised and cultured on rooting medium for rhizogenesis (Figure 2D).Total number of 652 explants was infected in seven sets of experiments, out of which 189 explants produced transformed shoots. Thus, transformation efficiency was calculated to be 16% - 44% (mean- 28.75 %),**(**Table 1**)**. After proper acclimatization, fully developed plantlets were hardened in soil rite (Figure 2E) and transferred to soil pots in glasshouse of National Phytotron Facility, IARI, New Delhi (Figure 2F).The presence of *Agrobacterium* in plant tissues was checked in transgenic lines by PCR using *Agrobacterium*-specific primers. All transgenic lines were free of *Agrobacterium* contamination as they did not show any bands by primer specific PCR reaction (data not shown).

Molecular Analysis of Transgenic *Brassica juncea* Cv. Varuna

PCR

Initially, the transgenic nature of the T0 plants was detected through PCR analysis. A total of eighteen kanamycin-resistant plants were subjected to PCR analysis with the specific primers for *Vigna radiata* lectin and *nptII* marker genes to confirm the stable gene insertion and expression of transgenes into the *Brassica juncea* host genome. Plants showing positive amplification of 828 bp specific to the *Vigna radiata* lectingene (Figure 3A) and a 728 bpspecific to the *nptII*marker gene (Figure 3B) analysis were subjected to segregation and southern analysis. No PCR band was observed with the non transformants (data not shown).

Semi-Quantitative RT-PCR Analysis

RT-PCR (Reverse Transcription PCR) was performed to check the expression of *Vigna radiate* lectin. The expression of lectin was analysed in leaf tissues by RT-PCR. Total RNA was isolated from the leaf tissue and its purity, concentration and integrity were determined. The A260/280 ratio of the isolated RNA samples was found to range from 1.4 to 2.0. The integrity of the isolated RNA was evaluated by performing electrophoresis of denatured RNA on agarose gel (1%) containing formaldehyde (Figure 3C). The presence of two prominent, distinct and intact RNA bands corresponding to 28S rRNA and 18S rRNA with no smearing indicated that RNA was of good quality. To normalize for sample to sample variation, β-actin gene was used as internal control (Suzuki et al., 2000) (Figure 3D). The total RNA was subjected to one step RT-PCR amplification using *Vigna radiata*lectin gene specific primers which were expected to give an amplicon of 828 bp (Figure 3E) and nptII specific primers to give an amplicon size of 728 bp (Figure 3F). Hence the presence of 828 bpamplicon would indicate the expression of*Vigna radiata* lectin gene in the T0 plants.

Segregation Analysis

T1 seeds were collected and grown on 1/2MS agar medium containing 50 µg/L kanamycin. Non transformant

seeds were also allowed to germinate in presence and absence of kanamycin. In the presence of kanamycin, non transformant seeds showed initiation of germination by the fourth day thereafter turned turned yellow and dried up, whereas the transformed seeds continued to develop as green seedlings. Eighteen putative transgenic plants were subjected to germinate in presence of kanamycin. All transgenic lines segregated in more or less similar ratio and co-inherited among the progeny plants following Mendelian pattern 3: 1 on kanamycin containing medium, (Table 2). However, one transgenic line showed the deviation from the Mendelian pattern 3: 1. The deviation may be related to multiple copy of transgene integration into host genome. Multiple copy insertion has been reported in transenic tobacco (Finneagan& McElroy, 1994).

Southern Analysis

Eighteen PCR-positive plants of T1 generations were subjected to Southern blot analysis using *Vigna radiata* lectin probe to confirm the integration of the transgene into the *Brassica juncea* genome. Consequently, the number of bands obtained on autoradiogram indicates the number of independent events of transgene integration. No signal was detected in non transformants. Out of eighteen PCR positive plants,five transgenic plants MVL4, MVL7, MVL 9, MVL27, and MVL29 showed a single locus for *Vigna radiata* lectin gene integration in the host genome, (Figure 4).

Aphid Bioassay

Aphid bioassay was used to study the insecticidal activity of *Vigna radiata* lectin gene expressed in transgenic plants. The survival of aphid was monitored with second instar nymphs an interval of 24 h for 7 days on T1 progenies of transgenic plants as well as non transformed plants.The difference was observed in mortality rate and reduction in fecundity of aphids in transgenic and non transformant plants after 3 days and it was found to be statistically significant. After 7 days, MVL7 and MVL29 line of T1 plant exhibited highest mean mortality of 22.66 % as compared to the non Transformants, (Graph 1). The mean mortality percentage of T1 putatives was found to be significantly ($p \geq 0.5$) different from non transformants after 7 days of assay period. As reported earlier, transgenic potato plants expressing GNA showed an enhanced resistence to peach potato aphid (Gatehouse *et al*., 1996) and potato aphid (Down *et al*., 1996). Also ASAL when were added in artificial diet or expressed transgenically in plant systems, they were shown to resist the attack of the sap-sucking insects including aphids, brown plant hopper, and green leafhopper (Dutta*et. al*. 2005, Wu. *et al*. 2006, Sadeghi*et al* 2007). Being of plant origin, lectin genes have high degree of compatibility with the metabolic system of transgenic host plants and are expected to give sustained protection against sap sucking insects.

CONCLUSIONS

The present findings demonstrate successful transformation of *Brassica juncea*cv. Varunausing *Vigna radiata* lectin under the control of phloem specific (*rolC*) promoter. Thus the developed transgenic will reduce the loss due to aphid which in turn will increase the production and lower the use chemical pesticide leading to improvement in farmer's economy.

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APPENDICES

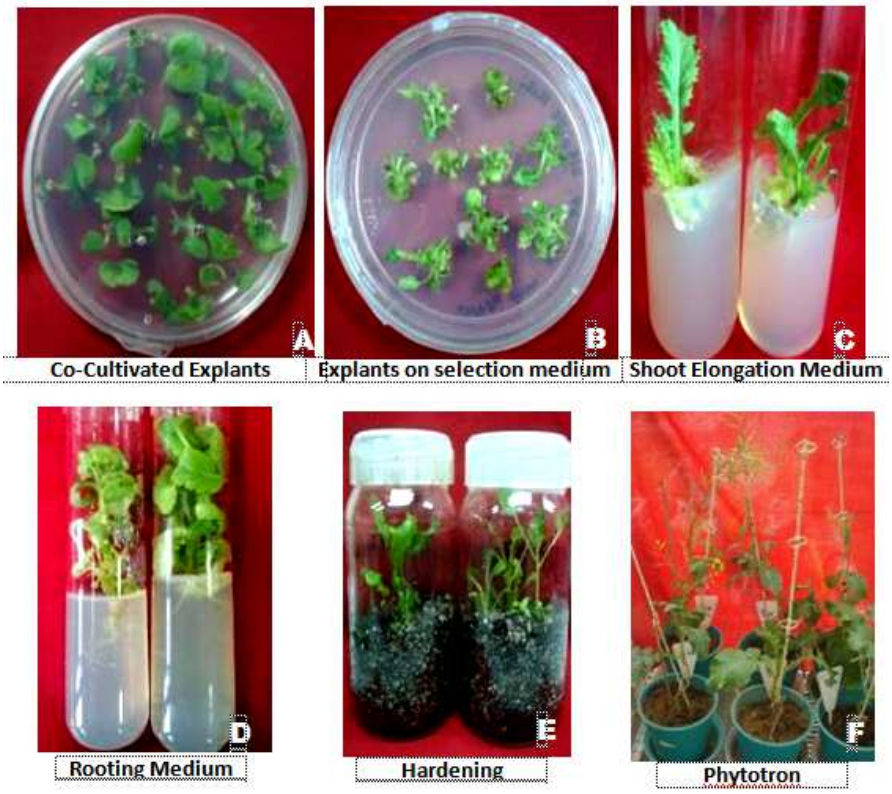

Figure 2: Transformation of *Brassica juncea* Cv. Varuna Using *Vigna radiata* Lectin Gene under Phloem Specific (rolC) Promoter

Table 1: Transformation Efficiency of transGenic *Brassica juncea* cv. Varuna

Batch No.	No. of Explants in co-Cultivation Medium	No. of Explants Transferred in Selection Medium	No. of Explants Showing Green Shoot Formation	No. of Plants in Rooting Medium	Transformation Efficiency %	No. of PCR Positive Plants	No. of Southern Positive Plants
1	98	68	16	7	16.32	1	-
2	76	50	17	4	22.36	2	-
3	87	23	12	3	13.79	1	-
4	99	75	28	9	28.28	2	2
5	79	53	35	18	44.30	5	1
6	103	71	42	23	40.77	3	1
7	110	89	39	16	35.45	4	2

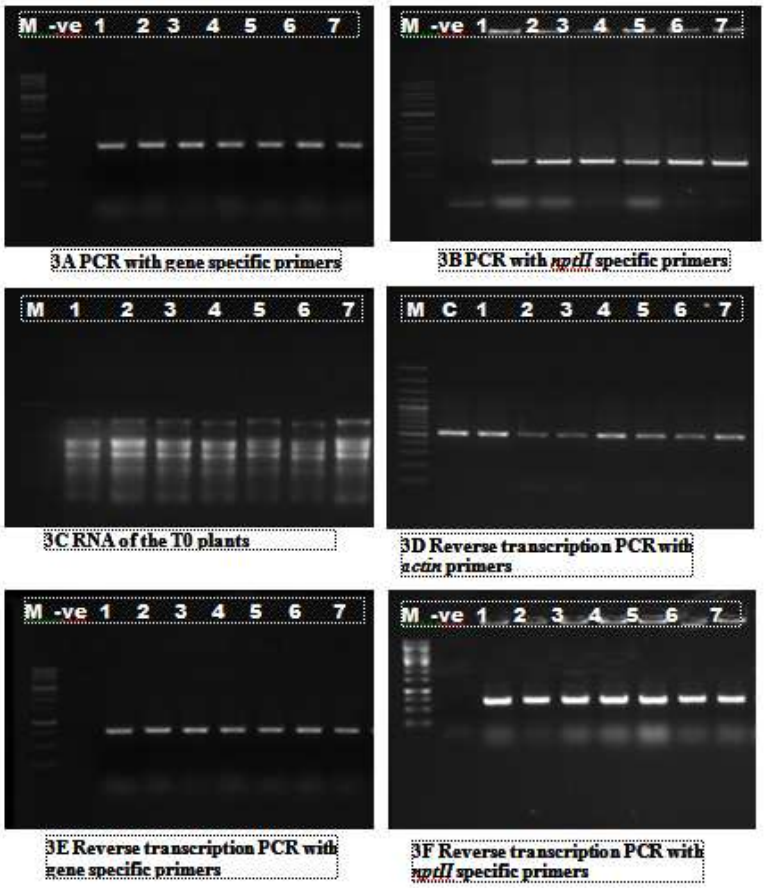

Figure 3: Molecular Analysis of Putative Transgenics: (A) PCR of T0 Plants with Gene Specific Primers; (B) PCR with *nptII* Specific Primers; (C) RNA of T0 Plants; (D) RT-PCR with *actin* Primers; (E) RT-PCR with Gene Specificprimers; (F) RT-PCR with *nptII* Specific Primers; (M Lane - 1kb Ladder; -ve Control- Non-Transgenic Plants; C- Control Plants)

Table 2: Segregation Pattern of the Kanamycin Resistant Gene in T1 Progenies

Sl. No	Plant Type	Selection in Presence/Absence Of Kanamycin (50 µg/L)	No. of T1 Seed Tested	No. of T1 Seed Germinated	No. of Seed Susceptible	Observed Ratio	Chi-Square Value (X2)	P Value
1	Control	(-)kan	70	66	-	-	-	-
2	Control	(+)kan	70	-	-	-	-	-
3	MVL4	(+)kan	50	38	12	3.1:1	0.006	0.938
4	MVL15	(+)kan	65	49	16	3:1	0.001	0.974
5	MVL7	(+)kan	59	45	14	3.2:1	0.012	0.912
6	MVL 9	(+)kan	46	35	11	3.1:1	0.007	0.933
7	MVL27	(+)kan	54	40	14	2.8:1	0.006	0.938
8	MVL25	(+)kan	62	46	16	2.8:1	0.005	0.943
9	MVL29	(+)kan	68	52	16	3.2:1	0.019	0.890

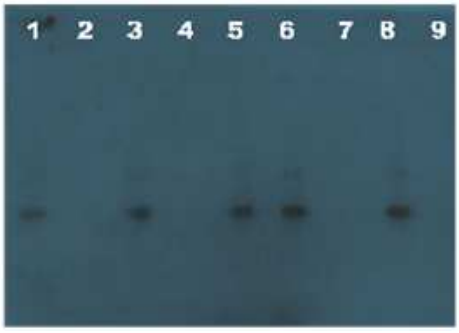

Figure 4: Southern Blot Analysis Oft1 Transgenic Plants Using *Vigna radiata* Lectin Geneprobe. Five Transgenic plants MVL4 (Lane 1), MVL7 (Lane 3), MVL 9(Lane 5), MVL27 (Lane 6), and MVL29 (Lane 8) Showed a Single Locus for *vigna radiata* Lectin Integration in the Host Genome

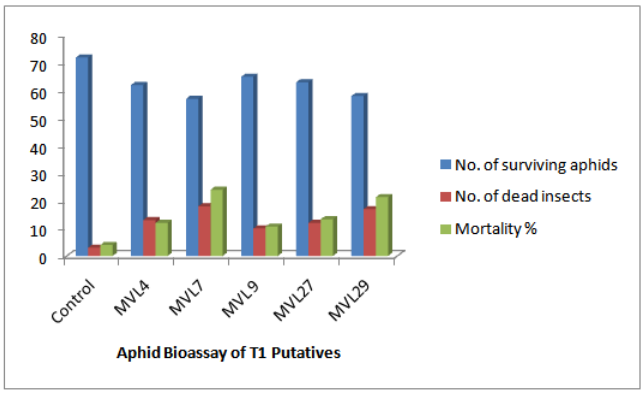

Figure 5

Graph 1- Aphid Mortality on T1 putativesincorporating gene constructs encoding *Vigna radiata* lectin.Twenty five second instar nymphal aphids in 3 replicates were placed on T1putatives as well as control plant leaf and aphid's survival was determinedafter 7 days. MVL7 and MVL29 line of T1 plant exhibited highest mean mortality of 22.66% as compared to the non Transformants. The mean mortality percentage of T1 putatives was found to be significantly ($p \geq 0.5$).